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Abstract

Human societies have converted biomass into energy and products for millennia using both biochemical and thermochemical processes. Familiar examples of biochemical processing includes fermentation of sugar- or starch-rich crops and milk into sauerkraut, beer, wine, yogurt, and cheese. Familiar examples of thermochemical processing include baking and cooking of food and burning wood for heat and power.

Keywords

biomass, gasification-based hybrid processing, microbial fermentation, pyrolysis-based hybrid processing, syngas fermentation, thermochemical deconstruction

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Hybrid Processing

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9.1 Introduction

Human societies have converted biomass into energy and products for millennia using both biochemical and thermochemical processes. Familiar examples of biochemical processing includes fermentation of sugar- or starch-rich crops and milk into sauerkraut, beer, wine, yogurt, and cheese. Familiar examples of thermochemical processing include baking and cooking of food and burning wood for heat and power.

Biochemical and thermochemical processes are also employed for the production of advanced biofuels and biobased products. The biochemical platform uses pretreatments and enzymatic hydrolysis to release reducing sugars from biomass followed by microbial fermentation to fuels or chemicals. The thermochemical platform uses heat and catalysts via the processes of pyrolysis, solvent liquefaction and gasification to produce liquid (bio-oil) or gaseous (syngas products), which are further upgraded to fuels and chemicals. Combining biochemical and thermochemical processes in a single system is known as hybrid processing, which provides unique opportunities for improving selectivity and efficiency in the production of fuels and chemicals [1, 2]. In general, hybrid processing encompasses a wide combination of biological, thermal and/or catalytic processes. In this chapter, we focus on the sequence of thermochemical deconstruction of biomass followed by biochemical upgrading to final products. Two prominent examples of hybrid thermochemical-biochemical processing are fast pyrolysis of biomass into pyrolytic substrates followed by microbial fermentation and gasification of biomass into synthesis gas (syngas) followed by syngas fermentation.

Hybrid processing captures the benefits of thermochemical and biochemical processes while mitigating their deficiencies. For example, thermochemical processing eliminates complex and costly pretreatment and enzymatic hydrolysis steps. It rapidly and economically converts the carbohydrate in whole biomass (cellulose and hemicellulose) into the fermentable intermediates irrespective of the biomass type and composition. For the pyrolysis-based hybrid processing, the thermochemical deconstruction of biomass can be performed in close proximity to biomass production sites to produce crude bio-oil suitable for transportation to a central upgrading facility. In this manner, low-density biomass is converted to high-density feedstock, reducing

transportation costs. With proper deconstruction and fractionation, lignin in the biomass is converted to a phenolic oil suitable for upgrading to drop-in hydrocarbon fuel [3], while the carbohydrate fraction of the biomass can be used as fermentation substrates for producing ethanol fuel and oxygenated chemicals [4]. For the gasification-based hybrid processing, biomass is converted into syngas, a uniform substrate for fermentation that includes carbon from both the carbohydrate and lignin content of biomass. Unlike Fischer-Tropsch (F-T) synthesis usually envisioned for processing syngas into fuels and chemicals, syngas fermentation does not require a strictly controlled hydrogen-to-carbon monoxide ratio and the biocatalysts are more tolerant sulfur and chloride contaminants in syngas than are metal catalysts. Syngas fermentation is also more selective of desired products than F-T synthesis [5, 6].

Although the research on syngas fermentation [7] and microbial utilization of bio-oil components [8] dates back to the early 1990's, the categorization as hybrid processing was first proposed in 1999 by Brown [9]. To date, the hybrid process based on fast pyrolysis of biomass followed by fermentation of pyrolytic substrates has been studied by relatively few researchers [1, 4, 10]. The gasification-syngas fermentation route has been more widely studied, including research on gas-to-liquid mass transfer [6, 11], strain development [12, 13] and commercialization [14-16]. This chapter explores the unique features, technical challenges, and future perspectives of these two approaches to hybrid processing.

9.2 Thermochemical conversion of lignocellulosic biomass for fermentative substrates

9.2.1 Fast pyrolysis for production of pyrolytic substrates

Fast pyrolysis of lignocellulosic biomass is the thermal decomposition of biomass in the absence of oxygen. This process produces an energy-rich liquid (bio-oil), a flammable gas mixture (syngas), and a carbon- and nutrient-rich solid (biochar) [17, 18]. The composition of bio-oil varies depending on biomass properties and pyrolysis type and operating conditions. An example of bio-oil composition from woody biomass (based on dry weight of biomass) is as follows: 15 wt% carboxylic acids, 25 wt% sugars, 4 wt% alcohols, 10 wt% aldehydes, 2 wt% esters, 7 wt% furans, 5 wt% ketones and 20 wt% aromatics [19]. Some of these compounds can be used as substrates for microbial fermentation [1, 4], which are discussed below.

9.2.1.1 Pyrolytic sugars

Pyrolytic sugars are produced from depolymerization of cellulose and hemicellulose during fast pyrolysis [1, 20]. The major sugar product of the fast pyrolysis of cellulose is the anhydrosugar levoglucosan (1,6-anhydro-β-D-glucopyranose) with small amounts of cellobiosan [21]. The yield of levoglucosan from cellulose can be as high as 60 wt%. The alkali and alkaline earth metals (AAEM, e.g. K, Ca and Mg) in biomass decrease levoglucosan yields due to the fragmentation of anhydrosugar rings, generating oxygenated compounds such as formic acid, glycolaldehyde, and acetol [22]. Mayes et al. [23] elucidated from the molecular level that the metal ions can decrease levoglucosan formation by up to 41.6 times, depending on the adjacency

of the cation to the reaction center. The major sugar products from fast pyrolysis of hemicellulose are xylose and dianhydrose xylose [24] . Acid washing of biomass to remove AAEM has been shown to increase anhydrosugar yield [25-29]. Alternatively, acid infusion to passivate AAEM through formation of thermally stable salts allows glycosidic bond cleavage to dominate pyranose and furanose ring fragmentation [30, 31]. Kim et al. [32] reported a sugar yield of 20.62 g/100 g biomass when acid-infused red oak was partially oxidized with nitrogen sweep gas containing 2.1 vol% oxygen.

9.2.1.2 Acetic acid

Acetic acid is the predominant carboxylic acid produced by deacetylation of hemicellulose during the fast pyrolysis of lignocellulosic biomass. In general, acetic acid is recovered in the aqueous phase of bio-oil [33, 34]. Due to its low heating value and corrosiveness, acetic acid is commonly regarded as an undesirable byproduct.

9.2.1.3 Lignin derivatives

Lignin is a major component in lignocellulosic biomass (15-30% by dry weight and 20-40% by energy density) and is responsible for the biomass structural integrity [35]. Because of the difficulty in its depolymerization and the heterogeneous nature of the depolymerization products, lignin valorization has long been a key challenge of traditional biochemical conversion processes [36]. During fast pyrolysis, lignin depolymerizes to various phenolic monomers and oligomers [37], which can be used as precursors for production of fuels and chemicals, although the subsequent repolymerization reduces their yield among the pyrolysis products [37, 38].

9.2.2 Gasification of biomass for syngas production

Compared to the fast pyrolysis, gasification of lignocellulosic biomass occurs at higher temperatures (usually 800-1000°C) with a limited amount of oxygen, producing syngas as the main product. Syngas is a gaseous mixture primarily comprised of 6-59 vol% carbon monoxide (CO), 29-76 vol% hydrogen (H_2) and 1-16 vol% carbon dioxide (CO_2), with a small amount of methane (CH_4). Depending on the biomass type and composition, the raw syngas also contains trace amounts of sulfur compounds (H_2S, COS), nitrogen compounds (NH_3, HCN), tars (i.e. condensable hydrocarbons), alkali metals, and chlorine [39]. Traditionally, syngas has been upgraded to alcohols and liquid hydrocarbon fuels through F-T synthesis, in which iron, cobalt, or ruthenium are used as catalysts for converting the gaseous compounds into liquid products under 200-350°C [5]. F-T synthesis usually requires a strict H_2 to CO ratio and is vulnerable to CO_2; the metal catalysts have lower selectivity and are very sensitive to some of the impurities (e.g. sulfur) in the syngas [6].

9.3. Biological conversion of fermentative substrates into fuels and chemicals

9.3.1 Fermentation of pyrolytic substrates

9.3.1.1 Pyrolytic sugars

Some microorganisms can utilize levoglucosan as source of both carbon and energy (Table 9.1) [40]. Many of these microorganisms use levoglucosan kinase (*lgk*) to convert levoglucosan into glucose-6-phosphate (G6P), which then enters the glycolysis pathway [41]. The yield of the products produced from levoglucosan is similar to that based on glucose [42, 43], indicating that levoglucosan to G6P is not a rate-limiting step. Some workhorse strains such as *E. coli* can be engineered for direct utilization of levoglucosan. For example, Zhuang and Zhang [44] expressed a cDNA library of *lgk* from fungus *A. niger* CBX-209 in *E. coli*, although the resulting enzyme activity was only one-third of that in the wild strain. Dai et al. [45] expressed the *lgk* gene from yeast *Lipomyces starkeyi* YZ-215 in *E. coli* BL21 and the resulting recombinant *E. coli* strain could grow on levoglucosan. More recently, ethanologenic *E. coli* KO11 was engineered to express *lgk* from *L. starkeyi* YZ-215; the engineered strain was able to use levoglucosan as a sole carbon source for ethanol production without additional antibiotics or inducers [41].

Table 9.1 *Microbial utilization of levoglucosan as carbon source (adapted from reference [2])*

Microorganism	Key enzyme	Intermediate	Product	Reference
Aspergillus terreus K-26	Levoglucosan kinase	Glucose-6-phosphate	Itaconic acid	[46]
Aspergillus awamori [a]	Levoglucosan kinase	Glucose-6-phosphate	N/A	[47]
Arthrobacter sp. I-552	Levoglucosan dehydrogenase	Glucose	N/A	[48]
Aspergillus niger CBX-209	Levoglucosan kinase	Glucose-6-phosphate	Citric acid	[49]
E. coli, expressing cDNA of *lgk* [b] from *A. niger* CBX-209	Levoglucosan kinase	Glucose-6-phosphate	N/A	[44]
E. coli BL21, expressing *lgk* from *Lipomyces starkeyi* YZ-215	Levoglucosan kinase	Glucose-6-phosphate	Ethanol	[45]
E. coli KO11, expressing *lgk* from *L. starkeyi*	Levoglucosan kinase	Glucose-6-phosphate	Ethanol	[41]
Rhodosporidium toruloides *Rhodotorula glutinis*	Levoglucosan kinase	Glucose-6-phosphate	Lipids	[50]

a. Multiple strains were reported including *A. fonsecaeus; A. luchuensis; A. niger; A. oryzae; A. sojae; Cryptococcus albidus; Fusarium solami; Neurospora crassa; Penicillum citrinum; P. cyclopium; P. expansum; P. granulatum; P. griseolum; P. italicum; Rhizopus niveus; R. oryzae; Sporobolomyces salmonicolor*

b. *lgk*: levoglucosan kinase

In addition to being directly utilized, levoglucosan can also be acid-hydrolyzed into reducing sugars for yeasts and fungi to produce ethanol or lipids (Table 9.2). This method, however, can result in some sugar loss during the neutralization of the acid hydrolysate [51].

Table 9.2 *Microbial utilization of levoglucosan that was acid hydrolyzed into glucose (adapted from reference [2])*

Pyrolysis feedstock	**Post-pyrolysis treatment**	**Strain(s)**	**Product/ Yield**	**Reference**
Wood	Aqueous extraction: water-to-oil 1:2 Detoxification: Activated charcoal treatment Hydrolysis: 2% (v/v) H_2SO_4 at 100°C for 120min Neutralization: lime	Yeasts; Fungi	Ethanol 0.43 g/g substrate	[8]
Cotton cellulose	Hydrolysis: 0.2 M H_2SO_4 at 121 °C for 20 min Neutralization: $Ca(OH)_2$ to pH 6.0 Detoxification: 10% (w/v) absorbent diatomite Filtration: 0.45 µm membrane	*Saccharomyces cerevisiae*	Ethanol 0.45 g/g substrate	[52]
Softwood	Aqueous extraction: 62% (w/w) water Hydrolysis: 0.5 M H_2SO_4 at 125 °C for 44 min	*S. cerevisiae*	Ethanol 0.46 g/g substrate	[53]
Hardwood	Solvent extraction: ethyl acetate/biodiesel blends Hydrolysis: 0.5 M H_2SO_4 at 120 °C for 42 min Detoxification: 100% (v/v) activated carbon Neutralization: $Ba(OH)_2$ to pH 7.0 Filtration	*S. cerevisiae* *Cryptococcus curvatus*	Ethanol 0.47 g/g substrate Lipids 0.1 g/g substrate	[10]

Cellobiosan (1,6-anhydro-*β*-cellobiose) is another anhydrosugar in bio-oil [52, 54-56]. Lian et al. [57] characterized soil samples and isolated six microbial species that could utilize cellobiosan as sole carbon source: *Sphingobacterium multivorum*, *Acinetobacter oleivorans* JC3-1, *Enterobacter* sp SJZ-6, *Microbacterium* spx FXJ8.207 and 203, and *Cryptococcus* sp. Each of these organisms was able to use levoglucosan as sole carbon source. However, to date, direct microbial fermentation of cellobiosan into a fuel or chemical product has not been reported. Cellobiosan can be hydrolyzed into reducing sugars including glucose, levoglucosan and/or cellobiose [58] that can be potentially utilized as fermentative substrates. It has also been shown that exogenously provided beta-glucosidase enzyme can cleave cellobiosan into levoglucosan and glucose [59].

9.3.1.2 Acetic acid

Acetic acid is the predominant carboxylic acid in bio-oil and is produced from deacetylation of hemicellulose [20]. Some microorganisms can metabolize acetic acid to produce acetyl-CoA, a central intermediate for biosynthesis of a wide variety of compounds, including fatty acids [60]. A number of oleaginous yeasts (*Cryptococcus albidus*, *Cryptococcus curvatus*, *Yarrowia lipolytica*) [61-63] and microalgae (*Chlorella protothecoides*) [64] have been reported to produce

lipids through the utilization of acetic acid as the sole carbon source, with resulting lipid content as high as 55% (g/g biomass). The main lipids produced by these oleaginous strains are C16-C18 fatty acids, ready for biodiesel production. However, there are only a few studies on lipid production using pyrolysis-derived acetic acid [65-68]. Unlike pyrolytic sugar fermentation based on anaerobic glycolysis pathway, lipid biosynthesis from acetate is an aerobic process. Therefore, maintaining an appropriate dissolved oxygen level is important for the carbon metabolism [62, 63, 69]. Important engineering issues to consider include aeration efficiency and power consumption.

9.3.1.3 Lignin derivatives

Two approaches are commonly used in the microbial utilization of lignin and its derivatives. One approach targets transformation of lignin-derived monomers into a specific target product. For example, vanillin can be produced by a number of specialized microorganisms from aromatic molecules such as eugenol [70-74], isoeugenol [74-85], ferulic acid [86-98], and vanillic acid [99] or by solid-state fermentation of green coconut husk [100]. The other approach is to funnel various lignin derivatives through the central metabolism of a microorganism to a single metabolic node, such as acetyl-CoA, and tune the target product based off of industrial relevance [101].

A number of phenolic monomers [37] can be utilized by aromatic-catabolizing microorganisms to produce the central intermediates (e.g., catechol and procatechuate) via aerobic peripheral pathway [102]. These central intermediates are then subjected to O_2-dependent aromatic-ring cleavages by dioxygenase enzymes found in many bacteria and fungi [102]. This aromatic catabolism serves as a "biological funnel" to reduce the heterogeneity of the lignin derivatives [101]. Linger et al. [101] reported utilization of a natural aromatic-catabolizing bacterium, *Pseudomonas putida* KT2440, to medium chain-length polyhydroxyalkanoates (*mcl*-PHAs) from lignin-derived phenolic liquor. Therefore, *P. putida* KT2440 could be used as a biological platform to produce muconate (13.5 g/L) from diverse lignin-derived aromatic monomers (e.g. phenol, vanillin, ferulic acid) via protocatechuate and catechol branches of the *β*-ketoadipate pathway. The muconic acid was recovered with high efficiency (74%) and high purity (>97%) and then subjected to catalytic hydrogenation to produce adipic acid, the most commercially important dicarboxylic acid. The aromatic-catabolizing pathways in *P. putida* KT2440 can be further modulated to optimize the yield of the desired product via the central intermediates (pyruvate, succinate, acetyl-CoA). For example, Johnson and Beckham [103] attained a 5-fold increase in pyruvate production by replacing the protocatechuate *ortho* pathway in *P. putida* KT2440 with a xenogeneic *meta*-cleavage pathway from *Sphingobium sp.* SYK-6. These advances in biological utilization of lignin derivatives enhance economic viability of the hybrid processing approach [35]. More recently, Salvachúa et al. [104] reported that a bacterial consortium of *P. putida* strains, *Amycolatopsis sp.*, *Acinetobacter* ADP1 and *Rhodococcus jostii* were able to depolymerize lignin with high molecular weight and catabolize the resulting aromatic monomers simultaneously. This may enable biological utilization of lignin-derived

phenolic oligomers in bio-oil, thus alleviating aforementioned monomer-repolymerization issues. However, since biological upgrading of lignin derivatives depends on aromatic catabolism with oxygen consumption, developing cost-efficient aeration will be crucial for economic viability and scalability of lignin valorization.

9.3.2 Fermentation of syngas

9.3.2.1 Metabolic pathway in syngas fermentation

A variety of microorganisms are capable of performing syngas fermentation. Based on the end products, those microorganisms can be hydrogenogens (Table 9.3) and acetogens (Table 9.4). The hydrogenogens produce H_2 from proton reduction coupled with CO oxidation to CO_2, which is also referred to as the biological water-gas shift reactions [105]:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \rightarrow CO_2 + H_2$$

The above reactions are catalyzed by two key enzymes: nickel-CO dehydrogenase (Ni-CODH) and CO-induced hydrogenase [106, 107].

Table 9.3 *Hydrogenogenic microorganisms in syngas fermentation and the optimal culture conditions* [a] *(adapted from reference [2])*

Microorganism	T (°C)	pH	Genome sequence	Reference
Citrobacter sp. Y19	30-40	5.0-8.0	N/A	[108, 109]
Rhodopseudomonas palustris	30	7.0	N/A	[110, 111]
Rhodospirillum rubrum	30	6.8	Available	[107, 112]
Rubrivivax gelatinosus	35	7.5	Available	[113-115]
Caldanaerobacter subterraneus	70	6.8-7.1	Draft	[116]
Carboxydothermus hydrogenoformans	70-72	6.8-7.0	Available	[117]
Carboxydocella sporoproducens	60	6.8	N/A	[118]
Carboxydocella thermautotrophica	58	7.0	N/A	[119]
Thermincola carboxydiphila	55	8.0	N/A	[120]
Thermincola ferriacetica	57-60	7.0-7.2	N/A	[121]
Thermolithobacter carboxydivorans	70	7.0	N/A	[122]
Thermosinus carboxydivorans	60	6.8-7.0	Draft	[123]
Desulfotomaculum carboxydivorans [b]	55	6.8-7.2	Available	[124]
Thermococcus onnurineus NA1	80	6.5	Available	[125-127]
Thermococcus sp. strain AM4	82	6.8	Available	[128-129]

[a] Unless noted otherwise, CO was used as substrate and H_2 was the products

[b] CO and sulfate were reported as substrates. H_2 and H_2S were reported as products.

Acetogens are facultative autotrophs capable of $CO/H_2/CO_2$ metabolism via the Wood-Ljungdahl (WL) pathway [130, 131]. The carbonyl branch and the methyl branch are two branches of WL pathway [15]. The overall stoichiometry of the pathway is presented as:

$$2CO_2 + 4H_2 + ATP \rightarrow \text{Acetyl-CoA} + 2H_2O + n\text{ATP}$$

where *n* is the ATP conservation coefficient; acetyl-CoA serves as a central intermediate and an ATP source, which is utilized to produce metabolites such as acetate and ethanol, with the supply of electron donors (H_2 or CO).

Table 9.4 *Acetogenic microorganisms used for syngas fermentation, their optimal culture condition and end products (adapted from reference [2])*

Microorganism	Substrate	T(°C)	pH	Product(s)	Genome sequence	Reference
Acetobacterium woodii	CO_2/H_2, CO	30.0	6.8	Acetate	Available	[132]
Acetogenium kivui	CO_2/H_2, CO	N/A [a]	6.6	Acetate	N/A	[133]
Acetonema longum	CO_2/H_2	30-33	7.8	Acetate, butyrate	N/A	[134, 135]
Alkalibaculum bacchi	CO_2/H_2, CO	37	8.0-8.5	Acetate, ethanol	N/A	[136, 137]
Blautia producta/ Peptostreptococcus productus	CO_2/H_2, CO	37	7.0	Acetate	N/A	[138, 139]
Butyribacterium methylotrophicum	CO_2/H_2, CO	37	5.5-7.4	Acetate, ethanol, butyrate, butanol	N/A	[140, 141]
Clostridium aceticum	CO_2/H_2, CO	30	8.3	Acetate	Available	[142, 143]
Clostridium autoethanogenum	CO_2/H_2, CO	37	5.8-6.0	Acetate, ethanol, lactate, 2,3-butanediol	Available	[144-147]
Clostridium carboxidivorans P7	CO_2/H_2, CO	37	5.8-6.2	Acetate, ethanol, butyrate, butanol, lactate	Draft	[148-150]
Clostridium drakei	CO_2/H_2, CO	25-30	5.8-6.9	Acetate, ethanol, butyrate	N/A	[148, 151]
Clostridium formicoaceticum	CO	37	NA	Acetate, formate	N/A	[152]
Clostridium glycolicum	CO_2/H_2	37-40	7.0-7.5	Acetate	Draft	[153, 154]
Clostridium ljungdahlii	CO_2/H_2, CO	37	6.0	Acetate, ethanol, lactate, 2,3-butanediol	Available	[13, 155]
Clostridium magnum	CO_2/H_2	30-32	7.0	Acetate	N/A	[156]
Clostridium mayombei	CO_2/H_2	33	7.3	Acetate	N/A	[157]

Clostridium methoxybenzovorans	CO_2/H_2	37	7.4	Acetate	Draft	[158]
Clostridium ragsdalei P11	CO_2/H_2, CO	37	6.3	Acetate, ethanol, lactate, 2,3-butanediol	Under construction	[145, 159]
Eubacterium limosum	CO_2/H_2, CO	38-39	7.0-7.2	Acetate, ethanol, butyrate	Available	[160, 161]
Oxobacter pfennigii	CO_2/H_2, CO	36-38	7.3	Acetate, butyrate	N/A	[162]
Moorella thermoacetica	CO_2/H_2, CO	55	6.5-6.8	Acetate	Available	[163]
Moorella sp. HUC22-1	CO_2/H_2	55	5.8-6.2	Acetate, ethanol	N/A	[164]
Moorella thermoautotrophica	CO_2/H_2, CO	58	6.1	Acetate	N/A	[165, 166]
Thermoanaerobacter kivui	CO_2/H_2	66	6.4	Acetate	N/A	[167, 168]
Desulfotomaculum kuznetsovii	CO, sulfate	60	7.0	Acetate, H_2S	Available	[169, 170]
Desulfotomaculum thermobenzoicum subsp. thermosyntrophicum	CO, sulfate	55	7.0	Acetate, H_2S	N/A	[169, 171]
Archaeoglobus fulgidus	CO, sulfate	83	6.4	Acetate, formate, H_2S	Available	[172, 173]

[a.] NA: not available

9.3.2.2 Metabolic engineering of acetogens in syngas fermentation

Most acetogens produce acetate as the sole product (Table 9.4). Some organisms are also capable of producing products such as ethanol, butanol, butyrate, and 2,3-butanediol, but the yields are usually low. Native acetogens have been engineered to divert carbon flow to the desired products with improved yields. Table 10.5 summarizes the recent advances of genetic manipulation of acetogens. Among those strains, *Clostridium ljungdahlii* has been widely used as a platform for heterologous gene expression. Six butanol pathway genes from *C. acetobutylicum* were expressed in *C. ljungdahlii* via a pIMP1 plasmid-based shuttle vector, with up to 2 mM butanol produced by the recombinant *C. ljungdahlii* [13]. Leang et al. [174] reported a more efficient electroporation protocol to perform chromosomal gene deletion for *C. ljungdahlii* via double-crossover homologous recombination with suicide vector. With this toolkit, Banerjee et al. [175] adapted the *bgaR*-P_{bgaL} plasmid-based lactose-inducible system originally developed for *C. perfringens* to *C. ljungdahlii* for acetone production. The inducible system redirected the carbon and electron flow for biosynthesis of the desired products other than acetate, producing 13 mM acetone with 25% carbon yield based on syngas input. The same lab later transformed 8 genes (*thl*, *crt*, *bcd*, *etfA*, *etfB*, *hbd*, *ptb* and *buk*) encoding the key enzymes for butyrate pathway from *C. acetobutylicum* to *C. ljungdahlii* [176]. The authors inactivated *pta*-dependent acetate, *adhE1*-dependent ethanol, and *ctf*-dependent fatty acid synthesis pathways to increase the butyrate titer to 17 mM, with 68% carbon yield and 73% electron yield [176]. Genetic manipulation of other

acetogens has also been reported (Table 10.5). For example, LanzaTech reported up to 25.6 mM butanol produced from steel mill waste gas (mainly CO) by overexpression of butanol synthesis pathway genes from *C. acetobutylicum* in *C. autoethanogenum* [177]. LanzaTech researchers identified two key enzymes, 2,3-butanediol dehydrogenase (2,3-BDH) and NADPH-dependent primary-secondary alcohol dehydrogenase (CaADH), contributing to 2,3-butanediol production during *C. autoethanogenum* syngas fermentation [178]. CaADH was demonstrated *in vitro* to convert acetoin, acetone and butanone to 2,3-butanediol, isopropanol and 2-butanol, respectively. This indicates the potential of using *C. autoethanogenum* as a platform for producing higher alcohols (C3, C4). Straub et al. [179] increased the acetate titer of *Acetobacterium woodii* to 51 g/L by overexpression of the native genes encoding the four THF-dependent enzymes, phosphotransacetylase and acetate kinase.

Table 9.5 *Engineered acetogens in syngas fermentation, target products and metabolic engineering strategies (adapted from reference [2])*

Host strain	Products	Metabolic engineering strategy	Results	Reference
Clostridium ljungdahlii	Butanol	Plasmid overexpression of butanol synthesis pathway genes *thlA*, *hbd*, *crt*, *bcd*, *adhE* and *bdhA* from *Clostridium acetobutylicum*	2 mM butanol production from syngas	[13]
Clostridium ljungdahlii	Acetate	Deletion of the bifunctional aldehyde/alcohol dehydrogenases *adhE1* and *adhE2*	Increased acetate production (64.4 mM) at expense of ethanol (4.7 mM)	[174]
Clostridium ljungdahlii	Ethanol, acetone	Adapting the *bgaR*-P_{bgaL} plasmid-based lactose-inducible system developed for *Clostridium perfringens* to *Clostridium ljungdahlii*	25% carbon flux redirected to acetone (~13 mM) as a result of reduced acetate yield	[175]
Clostridium ljungdahlii	Butyrate	Integration of *Clostridium acetobutylicum* butyrate pathway genes *thl*, *crt*, *bcd*, *etfA*, *etfB*, *hbd*, *ptb* and *buk* at *pta* locus of chromosome, and inactivation of *adhE1* and *ctf* genes encoding CoA transferase	Up to 1.5 g/L butyrate production from H_2/CO_2 or CO/CO_2	[176]
Clostridium autoethanogenum	Butanol	Plasmid overexpression of butanol synthesis pathway genes *thlA*, *hbd*, *crt*, *bcd*, *etfA* and *etfB* from *Clostridium acetobutylicum*	25.66 mM butanol production from steel mill waste gas	[177]
Acetobacterium woodii	Acetate	Plasmid overexpression of genes encoding the formyl-THF synthetase, methenyl-THF-cyclohydrolase, methylene-THF dehydrogenase, methylene-THF reductase, phosphotransacetylase and acetate kinase	Increased acetate titer from H_2/CO_2 to 51 g/L	[179]

Overall, development of metabolic engineering strategies for acetogens is still in its infancy. Kopke et al. [13] reported low butanol titer of 2 mM by engineered *C. ljungdahlii*; by the end of the batch fermentation, butanol was no longer detected, possibly having been consumed by the cells. In another project, Ueki et al [176] developed a genetic knockout system for *C. ljungdahlii* to interrupt acetate kinase and CoA transferase. However, this attempt did not completely eliminate acetate and ethanol production; the majority of carbon flux in the WL pathway still flowed to acetate, indicating the existence of other unidentified genes controlling their metabolism. Further efforts in metabolic engineering of acetogens are needed.

9.4. Challenges of hybrid processing and mitigation strategies

9.4.1. Pyrolysis-fermentation process

9.4.1.1 Fractionation of bio-oil

Bio-oil fractionation is commonly used to enrich specific pyrolytic substrate(s) in a distinct fraction. Bio-oil can be phase-separate by addition of water to produce two fractions: a light fraction containing oxygenated compounds such as carboxylic acids, acetol, and aldehydes, and a heavy fraction containing lignin-derivatives [180]. Pollard et al. [33] reported a unique multi-stage fractionation system containing a series of condensers and electrostatic precipitators to recover bio-oil as five distinct stage fractions (SFs), with each fraction having different physiochemical properties. With this innovative system, over 85 wt% of the sugar (mainly levoglucosan) in the crude bio-oil was recovered in SF1 and SF2, whereas acetic acid-rich aqueous fraction was condensed in SF4 and SF5. Both the sugar-rich phase and the acetic acid-rich phase have been tested as fermentation substrates [4, 66, 67]. Conventional distillation usually causes polymerization of bio-oil due to thermal and chemical instability of several components in the bio-oil. Vacuum distillation at lower temperatures is more successful [181-183] but still generates distillation residue of little value. Zhang et al. [184] reported a more efficient fractionation process with no waste generated. The authors separated bio-oil by atmospheric distillation, followed by co-pyrolysis of the distillation residue into acetic acid, propionic acid, and furfural.

9.4.1.2 Toxicity of crude pyrolytic substrates

The toxicity of contaminants in crude pyrolytic substrate is a major challenge for pyrolysis-based hybrid processing. Crude pyrolytic substrate is highly heterogeneous, containing hundreds of chemical compounds [185]. Some of these can severely inhibit microbial biocatalysts. For example, organic acids, furfural, and 5-hydroxymethylfurfural (5-HMF) are well-known for inhibiting growth and fermentation of ethanologenic *E. coli* [186, 187]. Furanic compounds can deactivate cell replication, inducing DNA damage, and inhibit key enzymes in central carbon metabolism [188], while phenolics cause inhibition by altering permeability of cell membranes and/or generating reactive oxygen species [189]. Acetol, furfural, 5-HFM and phenolics are toxic to oleaginous microalgae [66-68]. In addition to the well-known mechanisms described above,

crude pyrolytic bio-oil also contains numerous unidentified compounds with unknown inhibitory effects [55, 67].

9.4.1.3 Detoxification of pyrolytic substrates

Detoxification is a promising method to increase the fermentability of pyrolytic substrates. Various physical and chemical methods have been developed to remove or reduce inhibitory compounds present in crude bio-oil or its fractions, including water extraction [190], air stripping [191], activated carbon adsorption and/or absorption [8, 10, 50, 52, 65, 66, 191], biochar adsorption [192], liquid-liquid extraction [191, 193], and alkali/overliming treatment [52, 193, 194]. Treatments can be combined into highly efficient detoxification processes. For example, Rover et al. [195] used hot water washes (80-90°C) to recover 93 wt% of the pyrolytic sugar in the heavy fraction of fractionated bio-oil, followed by alkali (NaOH) treatment to remove most of the acetol, furans, and phenolic compounds without sugar loss. Ethanologenic *E. coli* were able to grow on up to 2 wt% treated pyrolytic sugars with cell densities comparable to that obtained for glucose at the same concentration; in contrast, untreated sugars strongly inhibited *E. coli* growth beyond 1 wt% [195]. It should be noted that challenges still exist for implementing industrial-scale detoxification processes. For example, overliming procedures consume large quantities of base and acid, which increases processing cost. Gypsum ($CaSO_4$) formed during the overliming will precipitate with sugars at alkaline pH, reducing sugar yield [196]. Humbird et al. [197] reported that sugar loss during precipitation of gypsum can be as high as 13 wt%. Stanford et al. [198] recently reported the efficacy of resins to remove contaminants from py sugars, which can be adapted to simulated moving bed filters.

Microbial and enzymatic treatments are alternative approaches for bio-oil detoxification. In this approach, microorganisms and/or enzymes selectively degrade specific inhibitory compounds while leaving fermentable substrates intact [199]. For example, laccases and peroxidases are capable of degrading phenolics and other aromatics [200-203]. Microorganisms including fungi (*Trichoderma reesei*, *Coniochaeta ligniaria*) [203-206], bacteria (*Ureibacillus thermophaericus*, *Cupriavidus basilensis*, *Rhodopseudomonas palustris*) [207-209] and yeasts (*Issatchenkia occidentalis*, *Iris orienalis*) [210, 211] can also detoxify various toxic compounds. The microbial/enzymatic-based detoxification has been used to treat acid hydrolysate in the lignocellulosic biomass biochemical conversion platform to remove phenolic compounds [200, 202, 203], furfural and 5-HMF [204, 205, 208, 212], and carboxylic acids [210]. As most of the inhibitors derived from lignocellulose pretreatment and/or hydrolysis also exist in pyrolytic bio-oil [33], this method can be adapted to the detoxification of the pyrolysis product. For example, Khiyami et al. [213] reported detoxification of corn stover pyrolysis liquors by white-rot fungi *Phanerochaete chrysosporium* capable of secreting ligninolytic enzymes. The biological detoxification approaches have advantages of mild reacting conditions, elimination of extra complicated separation procedures and less waste generation. However, a long incubation period, high enzyme costs, and sugar loss are the challenges to be overcome [203].

9.4.1.4 Enhancing tolerance of microorganisms to toxic compounds in crude pyrolytic substrates

Another strategy for solving the toxicity problems in pyrolytic substrate fermentation is to enhance microbial tolerance to toxic compounds [214]. When the inhibition mechanism of a toxic compound is known, rational strain engineering can sometimes be used to increase tolerance. For example, ethanologenic *E. coli* possesses an NADPH-dependent furfural reductase capable of converting furfural to a less toxic product, furfuryl alcohol [215], but this reaction depletes the NADPH needed for cysteine biosynthesis, which is especially important when cells are grown in low-cost defined mineral salts media. Miller et al. [216] enhanced furfural tolerance of *E. coli* LY180 by silencing two NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*). These gene deletions decreased the ability of the cells to convert furfural to the less toxic alcohol, but the associated increase in NADPH availability resulted in increased growth and ethanol production in the presence of furfural. The tolerance of *E. coli* LY180 to furfural was also improved to 10 mM by plasmid-based expression of *thyA* gene from *Bacillus subtilis* [217]. Cell growth and ethanol production (30 g/L) were restored after an initial 48-h lag phase, during which time furfural was completely converted to less toxic furfuryl alcohol. As NADH represents a favorable alternative for reducing furfural to less toxic alcohol instead of NADPH, Wang et al. [218] overexpressed *fucO*, an NADH-dependent propanediol reductase, in *E. coli* LY180 to enhance furfural tolerance. The engineered strain could tolerate 15 mM furfural and produced ethanol at a titer of 45 g/L, comparable to the titer observed when cells were grown in furfural-free medium. Wang et al. [219] further investigated the epistatic interactions among the four beneficial genetic traits (*ΔyqhD*, *pntAB*, *fucO*, and *ucpA*) for furfural tolerance in *E. coli* LY180, leading to substantial biomass growth and ethanol productivity during xylose fermentation in the presence of 15 mM furfural.

Random mutagenesis, such as directed evolution, is another strategy to improve microbial tolerance to toxic compounds [66, 193]. This approach is more commonly used in pyrolytic substrate fermentation as the toxic compounds in crude substrate solution are very complex and the inhibition mechanisms are usually unknown. Directed evolution mimics natural evolution in the laboratory environment. Microorganisms are exposed to an environment containing small amounts of the toxic compounds so that they acquire mutations that improve growth in the environment. The concentration of the toxic compounds is gradually increased during the evolution process, and strains with beneficial mutations will eventually dominate the population. The mutant strains can then be characterized by, for example, DNA sequencing. A typical example of using directed evolution for enhancing a microorganism's tolerance of crude pyrolytic substrates is reported by Liang et al. [66], in which the microalga *Chlamydomonas reinhardtii* was cultured with acetic-acid-rich bio-oil fraction for producing lipid-containing biomass. After multiple generations, the evolved algal strain was able to grow in media containing substantial amounts of pyrolytic acetate.

9.4.2. Gasification – Syngas fermentation process

9.4.2.1. Inhibitory compounds in syngas and gas cleanup

Syngas produced by gasification of lignocellulosic biomass typically contains small amounts of sulfur gases (H_2S, COS), nitrogen gases (NH_3, NO, HCN), tars, and particulate matter (ash and char) [6]. Although syngas fermentation does not require as strict of clean-gas composition as metal-catalyzed Fischer-Tropsch synthesis, contaminants nevertheless can suppress product yield, or even cause process failure if contaminants exist in too high of concentration [220-223]. Indeed, it has been reported that INEOS Bio, the first commercial cellulosic ethanol producer based on syngas fermentation, experienced severe disruption in its process, resulting from high levels (~15 ppm) of HCN contaminant [224].

Syngas contaminants can be minimized by feedstock pretreatment and/or gasification process optimization. For example, Broer et al., [225] used steam/oxygen-blown gasification with optimized equivalence ratio (ER) to reduce contaminant production. Contaminants that formed were effectively removed though multiple gas-cleaning steps post-gasification [225]. Cyclones, electrostatic separation, and barrier filtration are commonly used in hot syngas cleanup, while water/liquid absorption is used for up cold-gas cleaning [39].

It should be noted that the composition of major syngas species (H_2, CO, CO_2) varies with biomass feedstocks and gasification conditions, which can impact performance of syngas fermentation. For example, excess H_2 was reported to promote ethanol production by *C. ljungdahlii* [226]. The electrons and protons required by the strain can be obtained from H_2 oxidation and/or CO oxidation/CO_2 reduction. Electrons from H_2 oxidation are preferred, so that CO can be utilized as a carbon source for metabolites synthesis rather than be sacrificed as an energy source for electron donation. In addition to H_2 content, the ratio of CO to CO_2 was found to affect syngas fermentation in certain strains such as *C. carboxidivorans* P7 [227].

9.4.2.2. Mass transfer limitation and bioreactor design

Gas-to-liquid mass transfer is a main bottleneck for syngas fermentation due to the low solubility of gas species such as CO and H_2 [11, 228]. Reactor configuration is crucial for enhancing the volumetric mass transfer coefficient (k_La). Continuous stirred tank reactor (CSTR) is the most commonly used configuration. Increasing the gas flow rate and agitation speed enhances k_La, but at high energy cost [229]. Other strategies to enhance k_La are the use of microbubble dispersion [230] and/or porous particles with large Brunauer, Emmett and Teller (BET) surface areas and specific surface properties [231]. Zhu et al. [232] reported an increase of 190% iin CO k_La using mesoporous silica nanoparticles. In *R. rubrum* fermentation, H_2 yield was improved by 200% when the medium was dosed with MCM41 functionalized nanoparticles [232]. Applying this strategy to commercial-scale syngas fermentation, however, is still challenging due to the high cost of purchasing and recovering these particles.

Bubble column reactors (BCR) can also be used in syngas fermentation. BCR can achieve high k_La values with low power consumption [233]. The maintenance and operational costs of

BCR are low. However, back-mixing and gas bubble coalescence may reduce mass transfer efficiency [234]. Using BCR, Chang et al. [235] reported CO k_La of 72 hr^{-1} with acetate productivity of 5.8 g/L/day in *Eubacterium limosum* fermentation. Rajagopalan et al. [236] achieved ethanol concentrations of 0.16 wt% and productivity of 1 g/L/day for syngas fermentation with *C. carboxidivorans* P7 [234]. A BCR containing a monolithic column improved ethanol production during syngas fermentation [237]. The monolith is composed of thousands of parallel micro channels separated by thin walls that support biofilm growth. Slug flow of gas and liquid inside each microchannel occurs under certain operating conditions [238]. Formation of a very thin liquid boundary layer sandwiched between the gas and biofilm greatly increases mass transfer efficiency [237].

Trickle bed reactors (TBR) have also been used for syngas fermentation. Klasson et al. [239] reported CO k_La value of 55.5 hr^{-1} in a TBR, considerably higher than reported for a packed-bed reactor (k_La 2.1 hr^{-1}). Orgill et al. [240] reported the effects of bead size, gas, and liquid flow rates on O_2 k_La in a TBR, achieving k_La of 421 hr^{-1} at liquid flow rate of 50 mL/min for 6 mm beads. The authors applied the same condition during semi-continuous fermentation of *C. ragsdalei* P11 and achieved 90% and 70% conversion of CO and H_2, respectively [241].

Hollow fiber membrane (HFM) bioreactor are promising for syngas fermentation, characterized by efficient mass transfer [240, 242-246]. Syngas is fed into the lumen of the HFM bundle and diffuses through the membrane. Cells growing on the outer wall of the membrane are able to consume dissolved gas and excrete metabolites into the medium. Mass transfer efficiency depends on numerous factors such as hydrophobicity, porosity, pore size, fiber diameter, and thickness of membrane material [240], internal or external placement of the HFM module [244, 245], membrane surface area [242], gas pressure [245], and gas flow rate through the lumen and liquid flow rate through the shell-side [242, 246]. Shen et al. [246] used HFM for continuous syngas fermentation of *C. carboxidivorans*, reporting a 10-fold improvement in CO k_La compared to a CSTR and achieved maximum ethanol productivity of 3.44 g/L/day. Major challenges in using HFM are fouling and loss of microbial viability during long-term operation. Cost-efficient strategies have been developed to maintain biomass viability and to minimize membrane fouling such as *in-situ* physical cleaning [247, 248].

Biologically active polymeric coatings represent an alternative approach to overcome mass transfer limitations in syngas fermentation. In this approach, microbial cells are concentrated and embedded in a thin, adhesive and nanoporous latex coating as an integral component of the bio-reactive structure. Cells are preserved under desiccated conditions, rehydrated to regain activity [249]. Applications include microbial photosynthesis, air pollution control [250-252], and more recently, syngas fermentation by immobilizing *C. ljungdahlii* on latex paper coatings [253].

9.5 Efforts in commercialization of hybrid processing

Pyrolysis-based hybrid processing remains in its infancy. However, recent progress in the production of pyrolytic sugar [254] and continuing advances in microbial engineering [255] give promise for the economical production of fuels and chemicals via fermentation of pyrolysis-derived sugars.

Gasification-based hybrid processing has a longer history of development, with several commercialization efforts emerging in the last several years [256]. For example, Coskata envisioned the steam reforming of natural gas to syngas followed by fermentation to ethanol It reportedly operated a demonstration plant in Madison, PA producing 40,000 gallons per year of ethanol before the company was sold to Synata Bio in in 2016 [257]. LanzaTech (www.lanzatech.com) entered a joint venture with the two largest steel companies in China to produce ethanol via microbial fermentation of CO-rich steel flue gas with a capacity of 300 million tons annually [14, 16]. Later, the company collaborated with Concord Blue (www.concordblueenergy.com) to produce ethanol and 2,3-butanediol (a jet fuel precursor) via gasification of woody biomass or solid waste (municipal solid waste, sewage sludge, agricultural residues) into high-quality syngas, followed by syngas fermentation. The pilot-scale facility began operation in 2015 to produce ethanol and value-added chemicals with a capacity of 250 L/day [258]. Another start-up company in syngas fermentation was INEOS Bio. They entered a joint Venture with New Planet Energy Florida known as INEOS New Planet Bioenergy, LLC, to construct a commercial plant near Vero Beach, FL [259]. The plant was designed to produce 8 million gallons per year of ethanol and 6 MW of renewable electricity via gasification of vegetative waste followed by syngas fermentation [260]. Unexpectedly high concentrations of HCN (15 ppm) in syngas caused production delays and eventually wet scrubber towers were installed to remove HCN to less than 1 ppm [261]. The company was sold to a Chinese investor in 2017 and renamed Jupeng Bio [262] while the biofuels plant was sold to Texas-based Frankens Energy LLC [263].

9.6 Conclusion and perspectives

Hybrid processing employs a thermochemical process, pyrolysis or gasification, to deconstruct biomass into fermentation substrates. Fast pyrolysis produces fermentable pyrolytic sugars, carboxylic acids and lignin derivatives, which can be utilized by microorganisms to produce advanced hydrocarbon biofuels. Currently, the inhibition from the toxic compounds is the biggest hurdle for the viability of pyrolytic substrate fermentation. Further research should focus on the development of more efficient detoxification methods for less toxic substrate and robust strains highly tolerant to toxicity. It is important to use both of the two strategies in order to enhance the economic feasibility of the pyrolytic substrate fermentation process. Moreover, it is necessary to elucidate the mechanism of toxicity so that a reverse engineering of evolved strains can be developed for an expanded genetic toolbox to be applied to other microorganisms.

Gasification-based hybrid processing is more advanced than the pyrolysis-based platform, having been developed over several decades. It is attractive for its ability to utilize both the carbohydrate and lignin in biomass feedstocks. Nevertheless, it faces several challenges to successful commercial development. Although many microorganisms are more robust to contaminants in syngas, gas clean-up is still required and can be both capital intensive and entail high operating costs. Gas-to-liquid mass transfer is rate limiting to the fermentation process and requires further improvement. Finally, the number of products from syngas fermentation are currently more limited than traditional sugar-based fermentations, which reduces market opportunities. For example, as the most prominent product of syngas fermentation, ethanol is not an optimal fuel due to its low energy density and the "blend wall" limitation [264].

Despite these challenges, hybrid processing remains an attractive approach for advanced biofuels and biobased products because of the robustness of thermochemical deconstruction of biomass and opportunities to improve fermentation of the resulting substrates through advances in biotechnology. Research directed toward these challenges in concert with growing demand for low-carbon fuels and biobased products will improve the commercial prospects of hybrid processing in the coming years.

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